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Molecular analysis of tube cement of the biofouling tubeworm Hydroides elegans

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14. ABSTRACT
Hydroides elegans is a common fouler that secretes a tube and strong cement. Little is known about the composition of either of these structures or the genes encoding them. We determined the amino acid composition of them. The ratios of the amino acids in the tubes and cements are different from other marine cements. We used next-gen. sequencing and determined that no marine-invert. cement homologs were found in the transcriptomes of the shell gland. We then used whole-mount in situ hybridizations to determine where highly expressed transcripts are expressed in the worms. Initial results show these transcripts are uniquely expressed in and around the shell and may be components of the cement.

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Biofouling, marine adhesives, tubeworm recruitment

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FINAL REPORT: GRANT # : N00014-12-1-0360
PRINCIPAL INVESTIGATOR : Michael G. Hadfield
INSTITUTION : Kewalo Marine Laboratory/ University of Hawaii
GRANT TITLE : A molecular analysis of tube cement of the tubeworm Hydroides elegans.
AWARD PERIOD : 3 June 2013 - 31 May 1858

OBJECTIVE : Due to the prevalence of *H. elegans* as a fouler in warm-water ports, and due to the strength of its cements, it is the objective of the proposed research to develop a much greater understanding of the chemistry of the *Hydroides* adhesive, both in its primary-tube phase, in which the worms first recruit (interacting with bacterial biofilms) on surfaces and in the calcified tubes in which the adult worms live.

APPROACH: Using a combination of advanced microscopy, transcriptomics, and molecular biology we will describe: (1) the major molecular components of the compounds upon which the tubes are constructed; (2) the precise location of the sources of the genes encoding these proteins in the worms via *in situ* hybridizations.

ACCOMPLISHMENTS:
The calcified tubes and cements of the biofouling tubeworm *Hydroides elegans* form the strongest surface attachment measured among common hard fouling organisms, and adhesion strength remains strong even when tubes are attached to commercial foul-release coatings. For this reason, understanding the nature of the cement produced by these worms is of considerable interest to those seeking to design surfaces to which the tubeworms cannot attach or can attach only weakly.
Cements of *H. elegans* are secreted during two separate stages of metamorphosis and growth. The first cements are secreted from the post-trochal region of settling larvae and is a component of a proteinaceous primary tube. This sticky tube is secreted by the larval epidermis and remains uncalcified. Secretion of the secondary, calcified tube is produced from a paired set of cement glands in the first thoracic segment of the metamorphosed juvenile. Protein preparations containing primary tubes and a second preparation of decalcified secondary tubes were created and amino acid analysis from acid hydrolysates of both of these preparations demonstrated that the matrices and the cements of the tubes may be similar to one another (Table 1).
Further, the amino acid composition of cements and tubes gathered from *H. elegans* was different than both the composition of foot proteins of *Mytilus californianus* and the tube cements of the polychaete *Phragmatopoma californica*. The tubes and cements from *H. elegans* also contain no DOPA residues. DOPA is an important component of the cements of *P. californica* and *M. californianus* and functions to crosslink and quinoine tan the cement proteins from both organisms. The lack of large percentages of this amino acid residue in the tubes and cements of *H. elegans* may suggest that a different molecular mechanism is used to harden the cements of *H. elegans.*
The secretion of the calcified secondary tube greatly increases the attachment strength of juvenile worms on both glass and commercially available foul-release surfaces. However, it is not known which genes may be involved in the production of the calcified secondary tube. Additionally, there are several secretory regions in the first segment of *H. elegans*

that may produce a precursor of the adhesive or the matrix that underlies the calcified tube, but it is unknown which tissues secrete tube matrix proteins. Several genes that have been implicated in the biocalcification of coral skeletons and mollusc shells and the transcriptomes of *H. elegans* were searched using BLAST for homologs of these genes (Table 2). PCR primers were designed for each of these genes from an adult sequences. PCR products were amplified from cDNA that had been reverse transcribed from RNA extracted from six adult worms that were actively secreting secondary tube matrix and cements.

Biocalcification Gene	**Function**
Calmodulin	Acidic Ca^{2+}-binding protein implicated in biomineralization in corals and bivalves
Galaxin	Part of calcifying matrix in corals and molluscs
Carbonic Anhydrase	Enzyme that converts CO_2 to calcium carbonate

Table 2. Biocalcification genes in *Hydroides elegans*

A 391 base pair (bp) homolog of a calmodulin gene was isolated from adult cDNA by PCR. This fragment was subsequently cloned into the pGEM-T easy vector and amplified in *E. coli.* BLAST was used to compare the sequence of this gene to those available in a protein database that is curated by the National Center for Biotechnology Information (NCBI). The amplified sequence closely matched a previously published calmodulin gene from *H. elegans*, and anti-sense riboprobes were constructed against this sequence. *In situ* hybridization techniques were used to visualize the pattern of expression in metamorphosing juvenile worms. The juveniles that were used for these hybridizations had begun to secrete both secondary cements and calcified tubes. Calmodulin transcripts are heavily expressed in the developing branchial rudiments of the juvenile and in epithelial cells located at the junction of the fore- and mid-guts of worms that had been fixed 4 hrs after the induction of metamorphosis. Calmodulin did not appear to be expressed in the shell gland nor the ventral epithelium of juvenile worms.

Galaxin is a secreted matrix protein that was originally isolated from corals and has subsequently been shown to be expressed by mantle tissue during shell deposition in molluscs. At least three galaxin pre-proteins are expressed in an adult transcriptome of *H. elegans.* A 710 bp fragment that showed sequence similarity a galaxin gene from the coral *Galaxea fascicularis* was selected for analysis of its expression patterns via *in situ* hybridizations. RNA riboprobes were generated against this sequence and *in situ* hybridization techniques were used to examine the expression patterns of this gene in newly metamorphosed juveniles of *H. elegans.* Surprisingly, this galaxin transcript was also not expressed in the shell glands nor in the ventral epithelium that underlies the collar. Instead, it segmentally expressed by the cells surrounding the setal muscles and in the unsegmented pygidium in the posterior of the juvenile worm .
In addition to calmodulin and galaxin transcripts, two carbonic anhydrase genes were cloned from cDNA generated from adult tissues of *H. elegans*. Carbonic anhydrases are enzymes that convert carbon dioxide to carbonante and these genes are thought to be required for biomineralization to occur. One of the carbonic anhydrases was 705 bp long and showed high sequence similarity to cytosolic carbonic anhydrases from the polychaete *Capitella teleta* and the mollusc *Lottia gigantea*. The domain architecture of this gene was analyzed and it contained a carbonic anhydrase domain but lacked either

transmebrane domains or a signal peptide domain. This architecture suggests that it is not secreted (lack of the signal peptide) and that it remains in the cytosol of the cell (no transmembrane domains). Labeled riboprobes were generated against this sequence and *in situ* hybridizations were performed against competent larvae (Fig. 2A) and juvenile worms that were six hours into the metamorphic process (Fig.2C). Cytosolic carbonic anhydrases were expressed in the collar of both competent larvae (Fig. 2B) and juvenile worms (Fig. 2D). The presence of these transcripts in the collar of both larvae and juvenile worms suggested that this gene may be involved in the biocalficiation of the secondary tube because juvenile worms use the collar to shape the calcified tube as it hardens. The increase in the intensity of labeling in collars of juvenile worms may also suggest that this transcript is up-regulated in these tissues. Further, cytosolic carbonic anhydrases are expressed in the mid- and hindguts of juvenile worms.

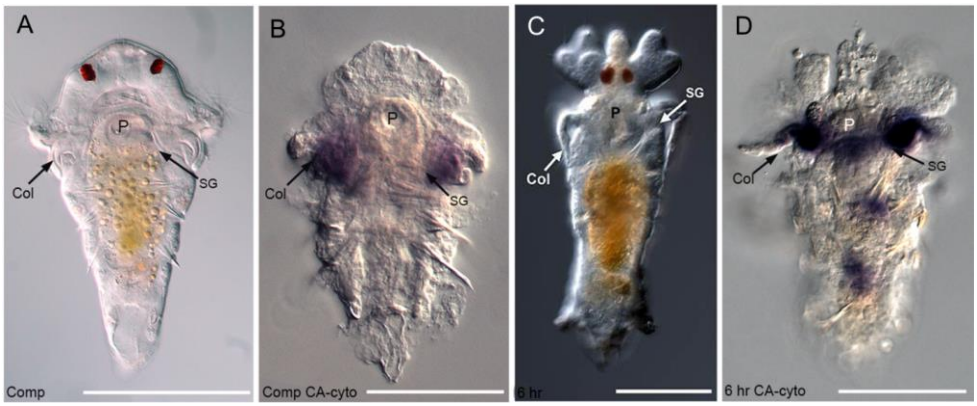

Figure 2. Expression of a cytosolic carbonic anhydrase in larvae and juveniles of *H. elegans.* A. Dorsal view of a competent larva. B. Expression of cytosolic carbonic anhydrase in acompetent larva. C. Dorsal view of a juvenile worm six hours after the induction of metamorphosis. D. Expression of cytosolic carbonic anhydrases in a juvenile six hours after the induction of metamorphosis. Col = collar, P= pharanx, SG = shell gland. Scale = 100 μm.

An additional carbonic anhydrase was cloned from cDNA of *H. elegans*. This transcript was 1271 bp long and showed high sequence homology to carbonic anhydrases (isoform –XIV) from *L. gigantea*. This isoform has several membrane-spanning regions and has an extracellular active site. This architecture suggests that this isoform catalyses the extracellular precipitation of carbonate. The domain architecture of this gene was analyzed and it contained a single carbonic anhydrase domain. Further, this transcript also contains four transmembrane domains and a signal peptide domain. This architecture suggests that this transcript was different from the cytosolic carbonic anhydrase and that it may be a transmembrane isoform. Riboprobes were generated against this gene and it too was expressed in the collar of both competent larvae (Fig. 3B) and juvenile worms (Fig. 3D). However, this expression was not as extensive as that of the cytosolic carbonic anhydrase (Fig. 2D). Transcripts for this transmembrane carbonic anhydrase were also expressed in cells at the base of the branchial rudiments (Fig. 3D). Because transmembrane carbonic anhydrases are expressed in the collar, they may also be a component of the biomineralization cascade of the secondary tube.

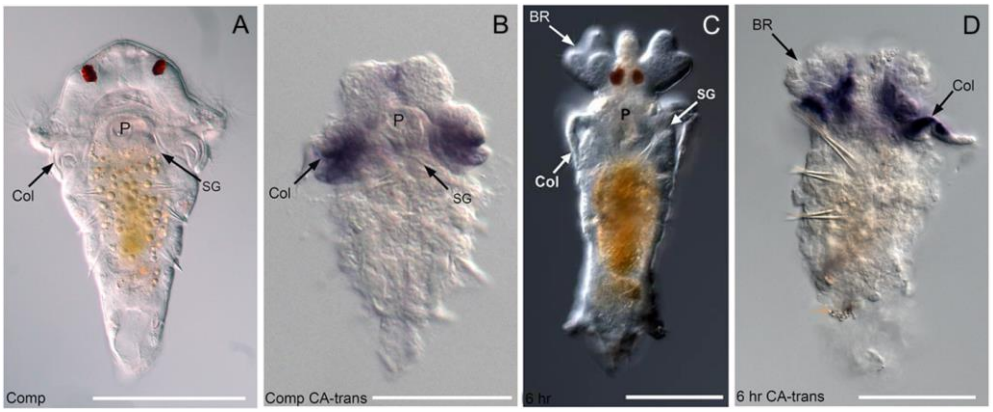

Figure 3. . Expression of a transmembrane carbonic anhydrase in larvae and juveniles of *H. elegans.* A. Dorsal view of a competent larva. B. Expression of transmembrane carbonic anhydrase in a competent larva. C. Dorsal view of a juvenile worm six hours after the induction of metamorphosis. D. Expression of transmembrane carbonic anhydrases in a juvenile six hours after the induction of metamorphosis. BR = Branchial rudiments, Col = collar, P= pharanx, SG = shell gland. Scale = 100 µm.

Tube cements from *H. elegans* are quite different than the adhesive cements produced by barnacles, mussels, and those of *P. californica.* The tube cements of *H. elegans* do not appear to be homologous to any other marine cements at either the genomic or protein levels. This lack of homology may make it difficult to identify potential cement genes because these transcripts will not have homologs in public databases and will be labeled as unidentified in any of our existing transcriptomes. In order to reduce the possible number of candidate genes, transcriptomes were generated from the first and second thoracic segments of adult *H. elegans*. These segments are morphologically similar, but the cement glands that are responsible for the secretion of adhesive cements lie within the first segment. The construction and comparison of two transcriptomes from morphologically similar tissues should reduce the number of unknown, differentially expressed gene-products and generate a realistic number of potential genes for examination. These transcriptomes were made from the first and second thoracic segments that were dissected from three replicate batches of 50 adult worms. Total RNA was extracted from each of the pooled samples and this RNA was shipped to the University of Utah's Genomics CORE for mRNA isolation, hiSEQ Illumina sequencing. The bioinformatics programs Trinity and Bowtie were used to create *de novo* assemblies from the raw sequencing reads (over 400,000) provided by Utah's CORE facility and these short sequences were assembled into 46,000 contigs (Fig 4D). Then, these contigs were assigned to the transcriptomes from either the 1st or 2nd segments. Next, the bioinformatics package EdgeR was used to determine the transcripts that were differentially expressed and upregulated in the transcriptomes created from the 1st thoracic segment and cement glands (Fig 4D). Approximately 3% of all transcripts (1,380) were differentially expressed and upregulated in the 1st segment (Fig 1E). BLAST was then used to annotate this pool of transcripts. Approximately 45% of these sequences (581) shared a significant sequence homology ($p < 0.001$) with proteins in the NCBI and EMBL databases and could be annotated. No published cement genes were present in the annotated portion of this transcriptome.

Because these cements are potentially novel and must be secreted by the cement glands in the first segment, a secretome was generated from the sequences of the 1st segment that

were a) up regulated and b) could not be annotated. A secretome is a set of transcripts that will be shunted to the endoplasmic reticulum for packaging and do not contain transmembrane domains. These proteins will be secreted into the environment. Hidden Markov models (HMMER) were used in concert with the bioinformatics programs SignalP and TMHMM to find the unannotated proteins that will be secreted. 111 transcripts were part of this potential secretome. Further, because we have counts of the number of times that contig appeared in one of the transcriptomes, we were able to sort the secretome and pick the top twelve contigs from this pool that had the highest levels of upregulation in the transcriptome from the first segment. PCR primers were designed for these sequences and PCR was PCR was used to amplify them from cDNA generated from the first segments of adult worms. After sequencing the PCR products to ensure that that the correct sequence was amplified, these sequences were subsequently cloned into *E. coli* and riboprobes were generated against these sequences. We then used these riboprobes and whole-mount *in situ* hybridizations to determine where RNA transcripts for these highly upregulated genes are expressed in competent larvae and post-metamorphic juveniles of *H. elegans.* Of the 12 contigs tested, only one gene (Orphan-1) was both highly upregulated in the first segment and expressed in the shell glands (Figs. 4F-I). Orphan-1 was and expressed in the shell glands and a small patch of the overlying collar in both competent larvae and juvenile worms. This pattern of expression suggests that it may be a component of the adhesive cement.

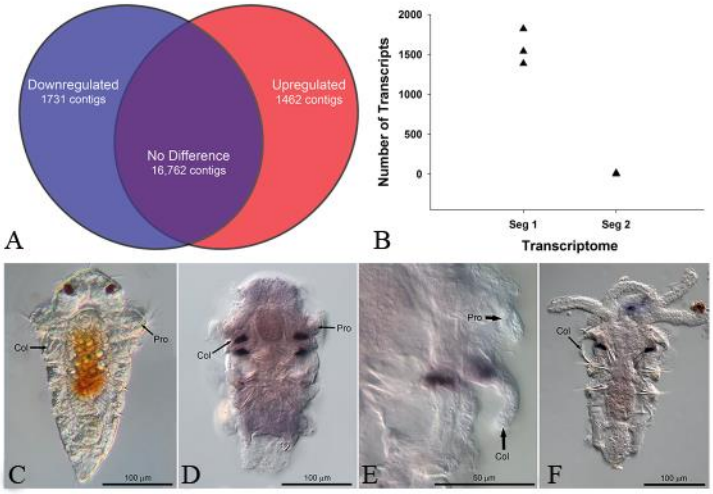

Figure 4. Analysis of tube cements of *Hydroides elegans*. A. Expression of transcripts in the transcriptome of the first (shell-gland) segment. B. The transcript Orphan-1 is highly upregulated in the first segment. C. DIC image of a competent larva. D. Pattern of expression of the transcript Orphan-1 in a competent larva. E. Close up of expression of Orphan-1 in collar region of a competent larva. F. Expression of Orphan-1 in the shell glands of a juvenile worm (24 hrs post-induction).

CONCLUSIONS *:*

The common biofouler *Hydroides elegans* uses a complex set of secretory tissues to produce two separate cements that it uses to attach itself to submerged surfaces. One of these cements is secreted by the post-trochal epithelium of larvae and it is used for the initial attachment to surfaces. A short time later, a second, different cement is produced by a paired set of glands located underneath the thoracic collar on the ventral surface of juvenile and adult worms. In addition to the temporal differences in the secretion of these

two cements, their amino acid composition is different from each other. These differences suggest that they are two separate cements.
Additionally, we determined that some of the genes that are part of the "biocalcification toolkit" for other metazoans are also expressed near the cement glands of larvae and juvenile worms. The gene products encoded by these genes may also be used by *H. elegans* produce its calcified secondary tube. The calcification of the secondary tube greatly increases the force required to remove it from both coated and uncoated surfaces. We have also used next-generation sequencing techniques to prospect for the genes encoding these cements in *H. elegans.* None of the known genes encoding for marine cements in other invertebrates are expressed in tissues of *H. elegans*. Because the cements of *H. elegans* show no homology to any other known cements, we assembled and examined the secretome of the segment of adult worms that contains the cement glands. We found one transcript (Orphan-1) that is highly upregulated in these tissues and is expressed in the presumptive shell glands of both larvae and juvenile worms.

SIGNIFICANCE:
The cements secreted by *H. elegans* appear to an evolutionary innovation because they are markedly different than other known marine adhesives. One of these potential glues (Orphan 1) shows no significant homology to other marine cements but it is expressed in the presumptive cement glands. Gaining a greater understanding of the properties and structure of this cement can provide insight into how tubes of *H. elegans* adhere so tightly to boat hulls and other submerged surfaces.

PATENT INFORMATION : None

AWARD INFORMATION: recipient of a research grant from the Gordon & Betty Moore Foundation to study bacterial mechanisms of settlement induction in *Hydroides elegans.*

PUBLICATIONS and ABSTRACTS (abstracts attached):
1. Hadfield, M.G. and B. T, Nedved. (2016). The bacterial basis of larval settlement. Abstract for Society for Integrative and Comparative Biology, Jan. 2016.
2. Nedved, B.T., G. Batzel., and M. G. Hadfield. (2016) Molecular analysis of tube cement of the biofouling tubeworm *Hydrioies elegans*. Abstract for Society for Integrative and Comparative Biology, Jan. 2016.
3. Batzel, G, B.T. Nedved and M.G. Hadfield. (2016) Presence and localization of carbonic anhydrase genes in *H. elegans.* Abstract for Society for Integrative and Comparative Biology, Jan. 2016.
4 . Batzel, G, B.T. Nedved and M.G. Hadfield. (in prep) Expression and localization of two carbonic anhydrase genes in the serpulid polychaete, *Hydroides elegans*. (this paper is in final draft and will be submitted this month)

Society for Integrative and Comparative Biology

2016 Annual Meeting

Meeting Abstract

11-2 Monday, Jan. 4 08:15 **The Bacterial Basis of Larval Settlement** *HADFIELD, M.G.*; NEDVED, B.T.; University of Hawaii at Manoa; University of Hawaii at Manoa*
hadfield@hawaii.edu

Bacteria and archaea have profusely colonized the earth for more than 3.5 billion years, forming dense microbial biofilms on virtually all marine surfaces. The first evolving animals, arising around 700 million years ago, adapted to the microbial world in many ways. One major animal adaptation is the use of biofilm bacteria or their products as signals for recruitment by larvae from at least seven marine invertebrate phyla. We have investigated the settlement biology of one such species, the circum-tropical serpulid polychaete *Hydroides elegans*. In-depth studies on larvae of H. elegans have revealed that its metamorphically competent larvae settle selectively in response to specific biofilm-dwelling bacterial species. The strongly inductive bacterium *Pseudoalteromonas luteoviolacea* produces complex clusters of bacteriocins, multi-protein structures evolutionarily derived from phage-tail elements, which induce metamorphosis of H. elegans. However, at least three other bacterial species isolated from biofilms and found to induce settlement of *H. elegans* lack the genes for bacteriocins. Among these, *Cellulophaga lytica* produces abundant outer membrane vesicles (OMVs) in culture, and cell-free suspensions of OMVs induce metamorphosis of *H. elegans*. To determine whether it is compounds in the bacterial membrane that surrounds both intact cells and OMVs or OMV contents that induce settlement, we isolated major membrane lipopolysaccharides from *C. lytica* and purchased bacterial peptidoglycan and found them not to be inductive when tested separately or in combination. This suggests it is “cargo” in the OMVs that is the inductive element. Knowledge of the precise inductive elements will allow us to explore the mechanisms by which larvae respond with the profound developmental events of settlement and metamorphosis. .

Society for Integrative and Comparative Biology

2016 Annual Meeting

Meeting Abstract

23-3 Monday, Jan. 4 14:00 **Molecular analysis of tube cement of the biofouling tubeworm *Hydroides elegans*** *NEDVED, B.T.*; BATZEL, G.; HADFIELD, M.G.; University of Hawaii at Manoa; University of Hawaii at Manoa; University of Hawaii at Manoa*
nedved@hawaii.edu http://www.kewalo.hawaii.edu

The serpulid polychaete *Hydroides elegans* is a common member of the warm-water marine fouling community. Its calcified tubes can form a layer several centimeters thick on submerged surfaces. The secretion of this tube begins early in metamorphosis and continues throughout the life of the worm. A strong cement is co-secreted with the tube, and both the tube and the cement are produced by a ventrally-positioned pair of “shell glands” located in the first thoracic segment. Little is known about either the composition of this cement or of the genes that encode it. In an attempt to better understand these cements, we used RNA-sequencing to assemble and annotate transcriptomes from both the first (containing putative cement glands) and second thoracic segments (lacking cement glands) of adult worms. We further determined the pool of transcripts that were differentially expressed in the first segment. No marine-invertebrate cement homologs were found in this transcript pool. Because the gene products for the cements are secreted and may be novel, we assembled a group of transcripts that were upregulated in the first segment, did not align with any proteins in NCBI databases, and contained a signaling peptide sequence. We then used whole-mount *in situ* hybridizations to determine where the 25 most highly expressed transcripts are expressed in competent larvae and in juvenile worms (24h post-induction). Initial results show that some of these transcripts are uniquely expressed in and around the shell glands in both larvae and juvenile worms, and may be components of the tube cement. Assembling a full set of transcripts compromising the cements of *H. elegans* promises to reveal a novel glue that is secreted and can set in seawater. .

Society for Integrative and Comparative Biology

2016 Annual Meeting

Meeting Abstract

91-1 Wednesday, Jan. 6 10:15 **Presence and Localization of Carbonic Anhydrase Genes in *Hydroides elegans*** *BATZEL, G*; NEDVED, B.T.; HADFIELD, M.G.; University of Hawaii at Manoa; University of Hawaii at Manoa; University of Hawaii at Manoa*
batzelg@hawaii.edu

Carbonic anhydrase (CA) is a metalloenzyme responsible for catalyzing the reversible hydration of carbon dioxide into bicarbonate. The CA-gene superfamily consists of five unique families (that arose independently from one another), with vertebrate and invertebrate lineages occupying the alpha-CA family. Although the original function of &alpha-CAs appears to have been as a pH regulator, due to its ability to regulate bicarbonate production, it has also been co-opted for biomineralization processes in calcifying animals. Until now, no study has tested for the presence or expression of alpha-CAs in a calcifying marine annelid. The tube building polychaete, *Hydroides elegans*, is an ideal candidate for studying calcification, because it forms a calcium carbonate tube that adheres tightly to submerged marine surfaces. We have identified ten unique alpha-CA transcripts in transcriptomes from *H. elegans*, consistent with the eleven total isoforms found in a non-calcifying polychaete, *Capitella teleta*. Furthermore, we report the successful cloning, expression, and localization of a secreted and a cytosolic CA isoform, HeCA1 and HeCA2 in H. elegans. Transcripts of both genes were localized in the calcifying collar segment of competent larvae and juveniles, suggesting that a combination of externally and internally mediated calcification may be occurring. Finally, we constructed a model for protein evolution that supports extensive gene duplication events that took place within the alpha-CA family. .